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EXPERT REVIEWS

Targeting mucosal dendritic cells with microbial antigens from probiotic lactic acid bacteria

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The use of vaccines against infectious microbes has been critical to the advancement of medicine. Vaccine strategies combined with, or without, adjuvants have been established to eradicate various bacterial and viral pathogens. A new generation of vaccines is being developed using specific strains of Gram-positive, lactic acid bacteria and, notably, some probiotic lactobacilli. These bacteria have been safely consumed by humans for centuries in fermented foods. Thus, they can be orally administered, are well tolerated by recipients and could be easily and economically provided to large populations. In this overview, we focus on mucosal immunity and how its cellular component(s), particularly dendritic cells, can be specifically targeted to deliver immunogenic subunits, such as the protective antigen from *Bacillus anthracis* (the causative agent of anthrax). An antigen-specific immune response can be elicited using specific strains of *Lactobacillus acidophilus* expressing the protective antigen. A mucosal, dendritic cell-targeted approach increases the bioavailability of an immunogen of interest when delivered orally by *L. acidophilus*. This provides an efficiently elegant natural strategy and serves a dual function as an immune-stimulating adjuvant *in vivo*.

KEYWORDS: adjuvant • dendritic cell • M cell • macrophage • mucosal immunity • probiotic bacteria • T regulatory cell • targeting antigen by specific dendritic cell-binding peptides • Th17 cell • vaccine delivery

The mucosa is a moist tissue that comprises particular organs and body cavities covering the aerodigestive, oropharyngeal, gastrointestinal, urogenital tracts and the eye conjunctiva. The mucosa represents the site for the first dynamic interactions between microbes and the human host. Accordingly, a powerful and highly specialized innate, as well as an adaptive, mucosal immune system protects the mucosal membrane interior and surface from pathogens, such as *Neisseria meningitidis, Neisseria gonorrhoeae, Entamoeba histolytica* and *Shigella, Salmonella* and *Listeria* spp. [1,2]. Although the mucosal site normally tolerates associated commensal microbiota, specific immunity can be induced against invading pathogens in mucosa-associated lymphoid tissues (MALT) through the homing specificity of activated effector lymphocytes [3].

Mucosal immunity functions in a variety of ways to protect the mucous membranes, such as fending off invading pathogenic microbes, capturing unprocessed immunogens derived from observed food and, finally, regulating immune responses through selective, immune-effector cascades. Accordingly, the MALT comprises highly compartmentalized areas of immune tissue that is induced independently from the systemic immune system. These areas include the Peyer's patches, mesenteric lymph nodes (MLNs), appendix, solitary follicles in the intestine, tonsils and adenoids of the aerodigestive tract, all of which serve as critical components of the mucosal induction site wherein initiated immune responses take place. The MALT is populated with distinct T and B cells, macrophages and subsets of professional antigen-presenting dendritic cells (DCs).

It has been postulated that immunogenic antigens are captured by absorptive and specialized epithelial cells, such as 'membranous' or 'microfold' (M) cells. These antigens are directly taken up and processed by B cells,

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14. ABSTRACT

Using vaccines against infectious microbes has been critical to the advancement of medicine. Various vaccine strategies combined with or without adjuvants have been established to eradicate various bacterial and viral pathogens. A new generation of vaccines is being developed using specific strains of the gram-positive lactic acid bacteria, and notably some probiotic lactobacilli. These bacteria have been safely consumed by humans for centuries in fermented foods. Therefore, they can be orally administered, are well-tolerated by recipients, and could easily and economically provided to large populations. In this overview, we focus on mucosal immunity and how its cellular component (s), particularly dendritic cells (DCs), cab be specifically targeted to deliver immunogenic subunits like the protective antigen (PA) from Bacillus anthracis (causative agent of anthrax). An antigen-specific immune response can be elicited by using various strains of Lactobacillus expressing PA. A mucosal, DC tasrgeting approach increases the bio-availability of an immunogen of interest, when delivered orally by Lactobacillus. This provides an efficiently elegant natural strategy and serves a dual function as an immune-stimulating adjuvant in vivo.

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macrophages or DC subsets, which subsequently present antigen to T-cell subsets residing within the inductive site for initiating specific immunity. In addition, epithelial cells appear to be actively involved in immunological processes. These cells express Toll-like receptors (TLRs), enabling them to interact with commensal or microbial products, such as lipopolysaccharide (LPS), flagellin and CpG [4–9]. Such recognition of highly conserved microbial structures through TLRs initiates cellular signaling responses that subsequently mobilize the immune response. Epithelial cells also release critical cytokines, such as TGF-β, thymic stromal lymphopoietin (TSLP) and chemokines (e.g., CCL20), which attract or functionally modify monocytic lineage cells such as DCs [10,11]. Nevertheless, many questions concerning the interplay of commensal microbes with the cellular mucosal system, and how precisely epithelial cells transport materials from one milieu into another, remain to be addressed.

Functional properties of M cells

Microfold cells are initially restricted to epithelial layers associated with lymphoid tissues and the respiratory tract. These cells are morphologically distinguished from other epithelial cells [12–17]. The M cells, when compared with intestinal enterocytes, possess a disorganized and diminished apical brush border. Its membrane-anchored glycoprotein network (glycocalyx) is also decreased and digestive enzymes, such as sucrose isomaltase and alkaline phosphatase, are downregulated, all of which are located apically in enterocytes [18]. Although one can stain these cells for intermediate filaments, mucin-related epitopes and lectin-binding sites specific for *Ulex europaeus* l lectin, there are currently no universal M-cell-specific cellular markers readily available [19]. Currently, the cellular origin of M cells is unknown and remains controversial. However, intestinal enterocytes, goblet, enteroendocrine, Paneth and M cells can be derived from precursors residing in the crypts, which then differentiate and migrate into crypts from the baseline towards the villi tips or follicle-associated epithelial dome [20,21].

It was recently shown that integrins, particularly β1, expressed on the basal membrane of enterocytes, may also mediate the adhesion of bacteria such as *Yersinia enterocolitica* to M cells through the interaction of bacterial surface proteins and apical integrins expressed on M cells [22]. Functionally, M cells internalize immunogens and sample bacterial, viral and parasitic microorganisms from the lumen to then transport them to underlying lymphocytes, particularly DCs. Owing to their transport activity, M cells play a critical role as inducers of mucosal and systemic immunity. Moreover, they were also recently identified in intestinal villi wherein these cells also internalize bacteria. This indicates that these cells are actively sampling antigens as seen in the follicle-associated epithelium [23]. The capture of microbial products by M cells is reminiscent of phagocytosis, a characteristic commonly associated with macrophage and DC functions. And yet, the question to be addressed in the future is whether transporting material from one milieu to another is the only mechanistic activity limited to M-cell function. Perhaps these cells are also capable of processing and shedding material into their environment, as lysosome-like compartmentalizations have been detected with the lgp 120, DAMP markers or a proteolytic enzyme, such as cathepsin E. Further studies are required to reveal their exact function within the mucosal system and to determine how these cells cooperate with other mucosal cells to mobilize immunity.

DC biology

Dendritic cells are a complex, heterogeneous group of multifunctional antigen-presenting cells (APCs) which comprise a critical arm of the immune system [24–27]. The role of these cells has been repeatedly highlighted in cancer and infectious diseases [28,29]. Recently, there have been great insights into the origins of DC subsets [26] and their modulation by distinct cytokines from neighboring cells [30]. DCs differentiate into at least four pathways: Langerhans' cells (LCs), myeloid DC, lymphoid (L) DC and plasmacytoid cells (pDCs) [31]. Progenitors of DCs in bone marrow migrate via the bloodstream and then home towards the peripheral tissues to confront invading pathogens [25–27]. In such environments, DCs ingest antigens via several mechanisms that include phagocytosis [32,33] and receptor-mediated endocytosis [34]. DCs phagocytose, process and present immunogenic peptides to various T-cell types [35,36].

Immunogenic subunits of different microbes and those used for vaccine strategies elicit inflammatory cytokines that include IL-1β, TNF-α, and IL-6. These cytokines promote DC-subset activation, maturation and migration to lymphoid organs where they are programmed to target T-cell-rich areas [37]. DCs, such as LCs, undergo phenotypic and functional changes during their maturation and migration. These cells, when loaded with immunogenic peptides on their MHC molecules, downregulate CD1a, CCR6 and Ca^{2+}-dependent E-cadherin and then lose the capacity to capture foreign antigens [38,39]. Human $CD14^+$ progenitor DCs, which differentiate due to a combination of cytokines (e.g., GM-CSF, TNF-α, TGF-β, IL-4 or IL-15) released by neighboring cells (e.g., mast cells, endothelial cells, fibroblasts or keratinocytes), migrate into the skin and secondary lymphoid organs. Such differentiated DC subsets may also home within lymphoid follicles, where they reside as germinal center (GC) DCs [40,41]. These latter cells establish the cross-talk between T and B cells, which may lead to the stimulation and activation of active immunity [40]. DCs present processed immunogenic peptides to $CD4^+$ T cells that then become activated in conjunction with costimulatory signals. Activated DCs can also prime naive $CD8^+$ T cells or undergo apoptosis [42]. Activated T cells migrate

into B-cell follicles and interact with naive antigen-specific B cells [43]. T- and B-cell interaction results in the clonal expansion of B cells, which takes place in the plasma foci of the T-cell-rich area and in the germinal centers [43]. T- and B-cell dialogue in the germinal center might be influenced by germinal center DCs [44] and follicular DCs [41].

Currently, the origin of phenotypic and functional properties inherent among DC subsets and critical cytokines released in the microenvironments, which determine the fate of DC precursors, are under intense scrutiny. Thus, new data from various groups dealing with DC immunobiology will provide more information on these cells and their role in diseases and vaccine technology.

Mucosal DCs

Dendritic cells have been identified at mucosal interfaces, including the lamina propria, the subepithelium, a T-cell-rich zone of lymphoid tissue associated with the mucosa and draining lymph nodes. DCs residing in the subepithelial region of Peyer's patches express $CD11b^+CD11c^+CCR6^+$, whereas those cells within the T-cell-rich zone and the MLNs express $CD11c^+CD8\alpha^+CCR7^+$. Additionally, $CD11b^-CD8\alpha^-$ DCs are also found in the Peyer's patches, MLN and epithelium [45]. DCs located in or beneath the epithelium can capture and sample various bacterial antigens (probably via TLRs), which cross the epithelial layer through M cells [46,47]. In addition, it was previously shown that DCs within the lamina propria, recruited by chemokines released by epithelial cells, reach the gut epithelia expressing occludin and claudin-1 molecules. These latter molecules facilitate penetration of these cells into the tight junctions and epithelial cells. DCs subsequently extend their probing dendrites into the lumen to sample commensal or microbial immunogens [45,48,49]. The cells then migrate into the lymphoid follicles wherein processed immunogens are presented to B and T cells to initiate humoral (IgA) and T-cell immune responses. Data showed clearly that the MLN represent barriers preventing the microflora from gaining access to systemic compartments that would induce detrimental inflammatory immune responses. The MLN serve as inductive sites for secretory IgA (sIgA) that prevent microbial binding to epithelia or induce the elimination of commensal bacteria via phagocytic cells in the gut mucosa (FIGURE 1).

Such mechanisms of sampling and capturing microbial products are critical for maintaining cellular homeostasis and the immune regulation that may restrict it in the gut where microbes normally reside [48,49]. Moreover, new data clearly show that IgA class-switch recombination (CSR) is regulated by inducible nitric oxide synthase (iNOS), which is preferentially expressed by MALT DCs [50]. It was previously demonstrated that iNOS controls the T-cell-dependent IgA CSR by TGF-β receptor, whereas T-cell-independent IgA CSR is regulated by APRIL and BAFF, in which the latter is a B-cell-activating factor of the TNF family. Such a discovery sheds light on sIgA that is secreted solely by plasma cells in MALT but not in other lymphoid organs. Tezuka *et al.* elegantly demonstrated that iNOS is induced in a subset of MALT DCs through TLRs recognizing commensal bacteria [50]. These data may explain the critical role of mucosal DCs, predominance of IgA secretion in the MALT, and how gut homeostasis is established by such mechanisms. Furthermore, mucosal immunity has evolved various mechanisms to achieve tolerance against self- and plethoric microenvironmental antigens found in food and airborne pathogens. Such mucosal tolerance could be induced through spontaneous cell apoptosis, regulatory molecules and the induction of regulatory T (Treg) cells, which were reported more than two decades ago [51].

New studies show that various gut-associated lymphoid tissues (GALT) DC subsets play critical roles through their unique immunological capabilities. These involve not only induction of antigen-specific T-cell immunity but also conversion of peripheral $CD4^+$ T cells to T regulatory cells that occur primarily after oral exposure to antigen. Such immune regulation by gut $CD103^+$ DCs on mucosal sites may be associated with their capacity to metabolize vitamin A into retinoic acid (RA). This molecule, in combination with TGF-β, selectively downregulates costimulation but upregulates expression of CCR9 and $\alpha4\beta7$ on conventional T and Treg cells, inducing cellular gut tropism and accumulation of $Foxp3^+$ Treg cells preferentially in GALT [52–58]. Additional data from the Pulendran laboratory demonstrated that macrophages from lamina propria expressing immunoregulatory molecules, TLRs and spontaneously secreting IL-10 tune down the differentiation of Th1- and IL-17-producing T helper cells (Th-17 cells) but promote the establishment of Treg cells. These macrophages also contrasted the differentiation of Th-17 cells, which were induced by $CD11b^+$ lamina propria-resident DCs [59]. These and other data from various laboratories shed light on the fundamental immunological interplay at the mucosal site and how intestinal immune machinery responds promptly to pathogenic microbes but tolerates commensal bacteria and food antigens. Such an intriguing site of mucosal immunity provides a great platform for vaccines and adjuvants to trigger DC subsets that subsequently activate mucosal cellular immunity towards regulatory or antigen-specific B- and T-cell immune responses.

Mucosal vaccines & adjuvants

The use of various vaccine strategies against infectious diseases has been one of the more profound accomplishments of the medical community. There has been more than a 95% decline in morbidity and mortality with various childhood infections since the employment of vaccine technologies and their universal utilization. This is evidenced by the fact that there has been no smallpox cases reported in the world for more than three decades and, moreover, poliomyelitis has now

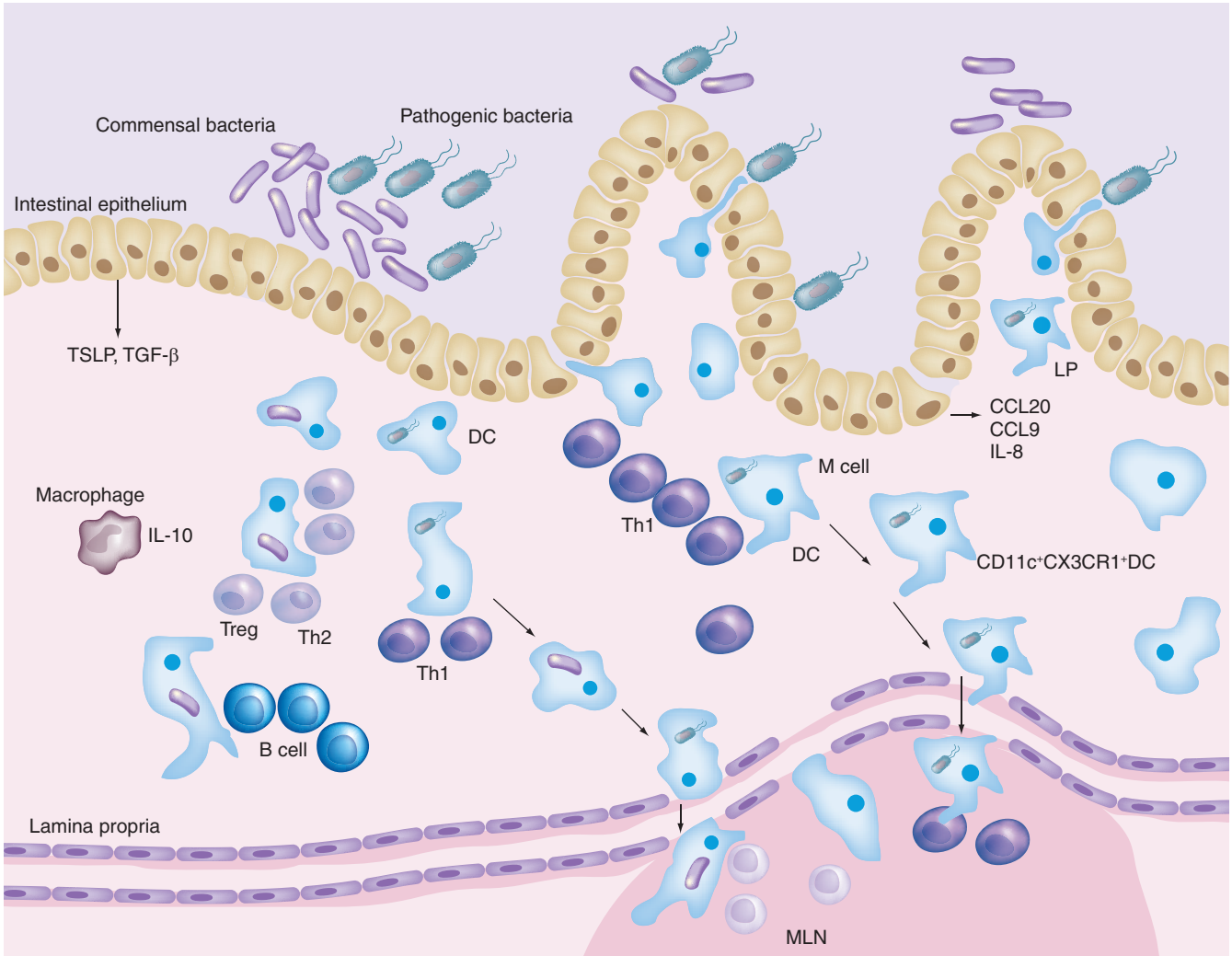

Figure 1. Sampling bacteria and their products by gut DCs. Various bacteria (e.g., pathogenic or harmless commensal bacteria) and their products in the gut lumen are captured by M cells and DCs. M cells of the gut epithelia import bacteria into the dome area of the gut-assiciated lymphoid tissue wherein DCs capture these bacteria (commensal bacteria) to elicit tolerance (Th2, Treg or B-cell class switching recombination) or protective immunity (Th1 responses) against pathogenic bacteria. Another route of capturing such bacteria is that DCs directly engulf them through their extended dendrites localized between epithelial cells of the gut lumen. Such engulfed bacteria will then be processed and presented to T and B cells either in the GALT, or within the mesenteric lymph nodes that drain the gut submucosa. DC-activated B cells subsequently produce bacteria-specific IgA that prevents bacterial migration into the gut mucosa that may trigger systemic inflammatory responses. T-cell activation or tolerance can also be achieved through macrophages and regulatory, or conventional, DCs.
DC: Dendritic cell; LP: Lamina propria; M cell: Microfold cell; MLN: Mesenteric lymph node; TSLP: Thymic stromal lymphopoitein.

been entirely abolished in Europe and North America. Thus, novel vaccine technologies and further refinement of exisiting methods and strategies attract talented scientists into the field. The establishment of mucosal vaccines, either for protection against microbes or for oral-tolerance immunotherapy, requires excellent antigen delivery and immune-modulatory adjuvants *in vivo*.

Therefore, vaccine strategies should protect the vaccine from enzymatic digestion and physical elimination that proves 'non-productive' and target specific mucosal professional antigen-presenting DCs situated in the inductive site to induce protective humoral and T-cell-mediated immunity. To date, various vehicles have been generated including insert systems such as liposomes and immunostimulating complexes (ISCOMs), live bacteria such as *Salmonella typhii*, and viral vector systems that include vaccinia or adenovirus [60–62]. Additionally, several live bacterial and viral vectors have been designed to promote cytokines that induce a microenvironment for cellular immunity *in vivo*. Promising results were recently achieved in both animals and humans for mucosa-targeting pathogens such as papillomavirus, calivirus, hepatitis E virus and filoviruses [63] by using nonreplicating virus-like particles [64–68].

However, none of these vaccines has to date been approved by the US FDA for human use. To enhance immunity *in vivo*, various potent mucosal adjuvants were recently used in several vaccine settings. Two of these immunomodulatory adjuvants are *Escherichia coli* heat-labile enterotoxin (LT) and cholera toxin (CT) B subunit [69,70]. These two toxins share 80% sequence homology and possess significant immune reactivity. CT interacts with M cells and binds to GM_1-ganglioside receptors of the epithelia. The adjuvant effects of CT, when combined with an immunogenic antigen, induce serum IgG and mucosal IgA humoral immune responses. CT and LT adjuvants can mono-ADP-ribosylate adenylate cyclase, which causes enzyme activation and elevated cyclic AMP levels that ultimately affect lymphocytes [71]. Nonetheless, it is critical to select an appropriate vaccination route to establish efficacy for any mucosal vaccine. One of the vaccine delivery routes for mucosal vaccination is oral administration, which upon the cellular components of GALT, provides robust protection against invading pathogenic microbes. Scientific efforts are now being mobilized by various groups to design mucosal vaccines through which professional antigen-presenting DCs can be reached, which in turn stimulate humoral and adaptive immune responses *in vivo*. To enhance bioavailability, a delivery vehicle and a potent adjuvant involving specific strains of lactobacilli can be selected. Thus, we review such potential delivery systems and highlight their beneficial application in vaccine strategies.

Lactobacilli as commensal bacteria in the GI tract

A number of *Lactobacillus* species are common members of the human gastrointestinal microbiota. Predominant among these are the homofermentative species *Lactobacillus acidophilus, Lactobacillus gasseri, Lactobacillus plantarum, Lactobacillus delbreuckii, Lactobacillus rhamnosus, Lactobacillus salivarius* and *Lactobacillus paracasei;* and the heterofermentative species, *Lactobacillus reuteri* and *Lactobacillus fermentum*. Species commonly found in both oral and fecal samples are *L. gasseri, L. paracasei, L. rhamnosus* and *Lactobacillus vaginalis* [72]. The highest levels of lactobacilli are typically found in the small intestine, where they can range from 10^4–10^6 colony-forming units/ml and form the predominant microbiota of that region. Historically, many of these *Lactobacillus* spp. have been consumed by humans in large numbers (e.g., ~$10^{7–8}$/g) as probiotic cultures, ingested either as dietary supplements or in more traditional fermented milk products, such as yogurt. Probiotics are defined as 'live microorganisms which when administered in an adequate amount, confer a health benefit to the host' [73]. Some of the benefits attributed to consuming probiotic cultures include protection against enteric pathogens and viruses, tolerance to lactose, anti-carcinogenic, anti-allergenic and modulation of the immune system [74]. It is widely accepted that these organisms play an important role as commensals in the GI tract. Their ability to survive transit through the stomach, close association with the intestinal epithelium, immunomodulatory properties, and their safe consumption at high numbers make lactobacilli attractive candidates for development of live vehicles for delivery of vaccines to the intestinal mucosa.

Lactobacilli serving as a potent adjuvant & efficient delivery vehicle in vaccines

The development of bacteria as live vaccine vehicles has focused primarily on the use of attenuated strains of pathogenic bacteria including *Samonella*, *Bortedella*, and *Listeria*

Table 1. Recent examples of lactobacilli as vaccine vectors.

Organism	Antigen	Model	Ref.
Lactobacillus casei	*Bacillus anthracis* PA	Mouse	[98]
	Clostridium tetani TTFC	Mouse	[83]
	Coronovirus glycoprotein S	Mouse	[99]
	HPV-16 E7	Mouse	[100]
	Salmonella enterica FliC	Mouse	[101]
	Streptococcus pneumoniae PsaA and PspA	Mouse	[102]
	SARS-CoV spike protein	Mouse	[103]
Lactobacillus helveticus	*S. pneumoniae* PsaA	Mouse	[104]
Lactobacillus plantarum	*Helicobacter pylori* UreB	Mouse	[105]
	S. pneumoniae PsaA	Mouse	[104]
Lactococcus lactis	*C. tetani* TTFC	Mouse	[81]
	Erysipelothrix rhusiopathiae SpaA	Mouse	[106]
	H. pylori UreB	Mouse	[107]
	HIV Env	Mouse	[108]
	HPV-16 E7	Mouse	[97]
	Plasmodium falciparum MSA2	Rabbit	[109]
	Rotavirus VP7	Mouse	[110]
	S. pneumoniae PsaA	Mouse	[104]

CoV: Coronavirus; FliC: Flagellin C; MSA: Merozoite surface antigen; PA: Protective antigen; PsaA: Pneumococcal surface adhesin A; PspA: Protective surface protein A; SARS: Severe acute respiratory syndrome; SpaA: Surface protective antigen A; TTFC: Tetanus toxic fragment C; UreB: Urease.

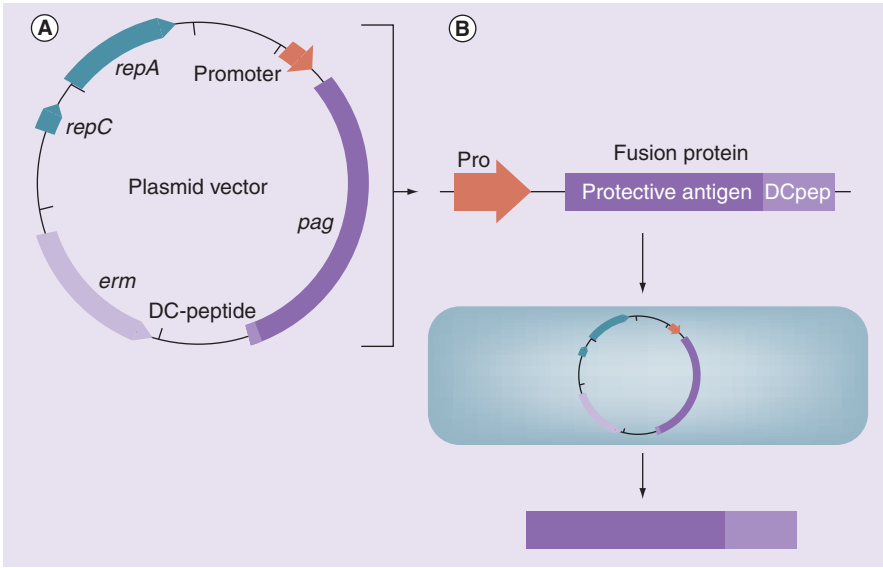

Figure 2. Expression of DC-targeted *Bacillus anthracis* PA in *Lactobacillus*. Specific 12-mer DC-binding peptides were discovered from a phage display peptide library. A DNA encoding sequence of DC-binding peptide fused to PA is expressed in *Lactobacillus acidophilus* NCFM using a specific plasmid-based system. **(A)** Schematic the PA–DC peptide or PA–control peptide constructs. **(B)** PA–peptide fusions will be expressed in *L. acidophilus* and secreted at mucosal surfaces for capture by DCs.
DC: Dendritic cell; PA: Protective antigen.

spp. [75–77]. While many of the properties related to their pathogenicity make them attractive candidates to promote immunogenicity, the potential for reversion of attenuated strains to virulence is a significant safety concern. Additionally, attenuated pathogenic bacteria are themselves highly immunogenic and this may prevent their use in vaccinations requiring multiple doses or with different antigens [78]. Due to their generally recognized as safe (GRAS) status and the availability of genetic tools for recombinant expression of proteins, specific species of lactic acid bacteria (LAB) are an attractive alternative to attenuated pathogenic bacteria. There is a growing body of work studying the potential use of live LAB in vaccine strategies [79, 80] where antigen-specific immune responses are induced *in vivo* (Table 1). A model antigen commonly used to evaluate the potential of LAB as vaccines was tetanus toxin fragment C (TTFC). Orally administered recombinant *Lactococcus lactis* [81], *L. plantarum* [82], *L. casei* [83] and *L. fermentum* [84] expressing TTFC elicited potent immune responses in mice. The ability of some LAB to persist, or not, in the GI tract may be critically important in the effectiveness of LAB-based vaccines. Accordingly, in a comparison of *L. plantarum*, a persisting LAB, and *L. lactis*, a nonpersisting LAB, Grangette *et al.* found *L. plantarum* to be more effective at eliciting antigen-specific immunity, suggesting that bacterial vector persistence in the GI tract impacted its immunogenicity [82]. However, further testing is needed to evaluate isogenic strains differing only in persistence capability or ability to associate intimately with the intestinal epithelium.

Nonetheless, it is important to use a LAB vector that can induce modulatory and stimulatory immune responses. It has been shown that LAB can mobilize a nonspecific, but balanced inflammatory response against infection, increase IgA production, activate monocytic lineages such as monocytes, macrophages, and DCs, and regulate the balance of Th1, Th2 [85] and, possibly, Treg cells. It is thought that probiotics influence the immune system, possibly by stimulating the expression of cytokines as shown *in vitro* [86–88] and *in vivo* [89]. Moreover, adjuvant-like effects on intestinal and systemic immunity have been demonstrated using LAB. Individuals consuming fermented milk containing *L. acidophilus* and *Bifdobacterium* species showed a fourfold higher specific serum IgA titer to *S. typhi* Ty21a than individuals not consuming fermented milk [90]. In addition, children receiving *Lactobacillus* GG during acute rotavirus infection showed enhanced IgA-specific antibody-secreting cell responses over those not receiving *Lactobacillus* GG [91]. Interestingly, it has been shown that particular *Lactobacillus* species induced critical inflammatory cytokines, activation and maturation of DCs [88]. It was also shown that immature DCs efficiently capture *Lactobacillus* species and these bacteria activated human DCs, resulting in proinflammatory cytokine production (e.g., IL-12), enhanced the proliferation of $CD4^+$ and $CD8^+$ T cells, and skewed these cells towards a Th1 pathway [92,93]. These observations highlight the ability of specific *Lactobacillus* species to promote DC activation, which in turn, regulates T-cell responses. Therefore, these consumable bacteria can naturally provide powerful adjuvant properties.

Current research

DC targeting

One promising strategy is the specific targeting of immunogenic antigens to DCs by probiotic microorganisms to elicit robust immune responses against the antigen of interest. This novel and highly innovative vaccine strategy provides various beneficial advances, including specific activation of DCs, directional elicitation of humoral and T-cell-mediated immunity by these cells, and a delivery system that can serve as a safe and potent adjuvant. To establish such a vaccine platform, a phage display peptide library was used to identify various 12mer DC-specific binding peptides [94]. To show the feasibility and efficacy of antigen targeting to DCs, genetic fusion of such DC-binding peptides to hepatitis C virus nonstructural protein (NS)3 showed significantly improved

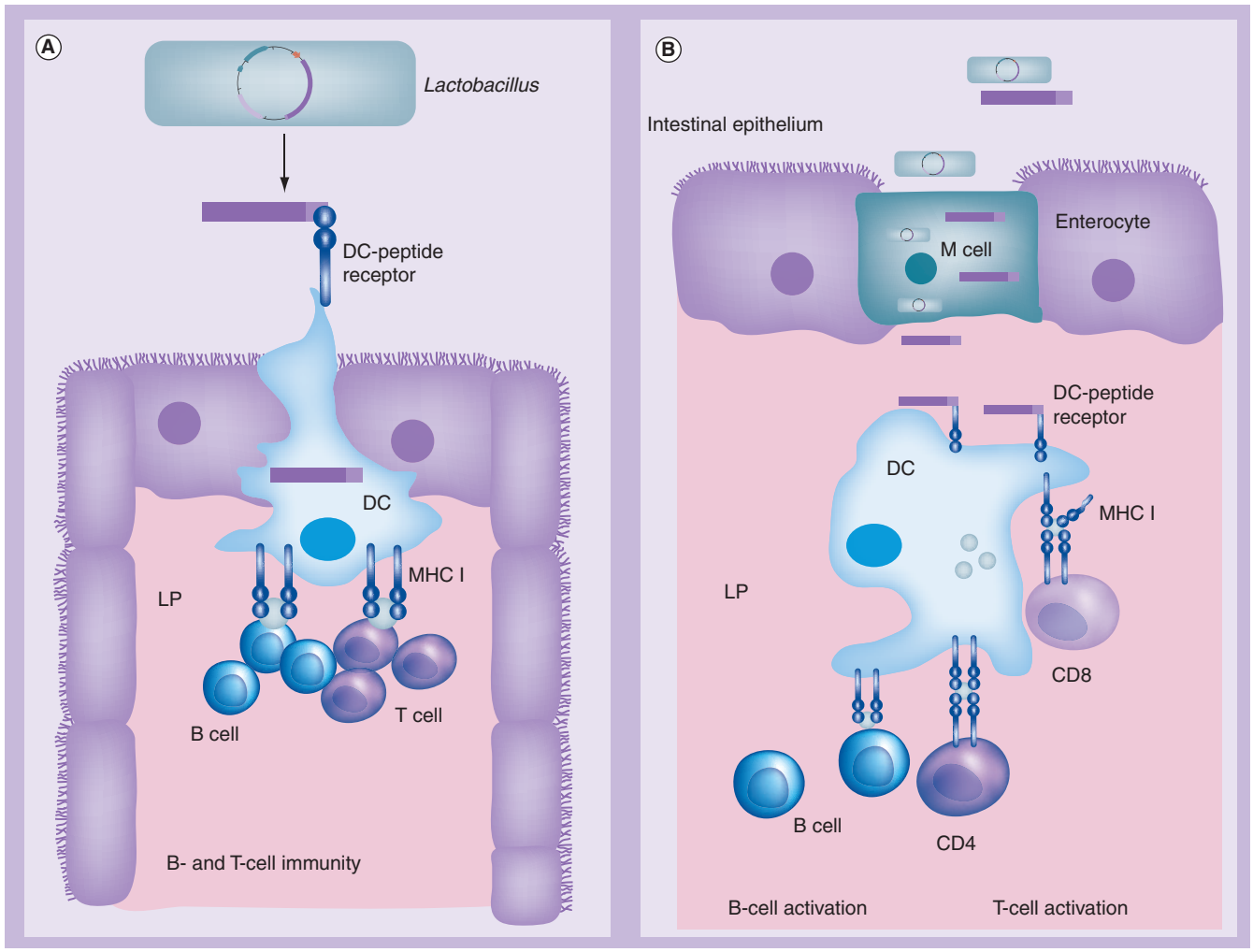

Figure 3. Delivery of immunogenic subunits to gut DCs by probiotic lactobacilli. (A) Engineered Gram-positive bacteria (e.g., *Lactobacillus acidophilus*) will be orally administered. These bacteria colonize the gut and release immunogenic fusion proteins into the gut lumen. DCs situated in the intestinal epithelia capture the immunogenic fusion protein through the moieties of DC-binding peptides expressed and released by lactobacilli. The DCs process, and then present it to B and T cells eliciting antigen-specific immune responses. **(B)** Alternatively, lactobacilli expressing immunogenic fusion proteins will be taken up by M cells and transported to gut DCs. These cells then internalize the bacteria and their contents including the immunogenic fusion. The processed immunogens will then be presented to T and B cells as peptides, eliciting protective immunity against disease and/or intoxication.
DC: Dendritic cell; LP: Lamina propria.

immunogenicity as compared with a NS3 control fusion protein or NS3 alone *in vivo*. We then designed a strategy to use probiotic bacteria to specifically deliver such an immunogenic fusion protein via *Lactobacillus* to mucosal DCs. DNA sequences encoding DC peptides were fused with antigen-coding sequences such as *B. anthracis* protective antigen and expressed in *L. acidophilus* (Figure 2).

Such engineered bacteria can be administered orally and flood the GI tract where, during transit, they secrete immunogenic fusion proteins into the intestinal lumen that specifically binds to its ligand expressed on mucosal DCs via DC-binding moieties (Figure 3A). In the case of nonsecreted proteins, lactobacilli expressing immunogenic fusion protein would be taken up by M cells and transported to gut DCs wherein immunogenic fusion proteins can be captured, processed and presented to T cells, inducing antigen-specific T-cell immune responses (Figure 3B). Data from our laboratories, and others, show promising results using this antigen-targeting approach to DCs, including *B. anthracis* protective antigen and botulinum toxin (heavy chain) *in vivo*. More studies are planned to optimize the dose and maximize the persistence of specific immune responses against biothreat agents.

Biocontainment of recombinant strains

The use of genetically modified organisms raises concerns about their release and propagation in the environment and the potential transfer of transgenes to other microorganisms. Steidler *et al.* described a strategy for biological containment

of genetically modified microorganisms [95]. A recombinant *L. lactis* strain auxotrophic for thymidine was made by replacing the thymidylate synthase gene (*thyA*) with a construct driving the expression of IL-10. The resulting strain produced therapeutic levels of IL-10 in cell cultures supplemented with thymidine, but died rapidly in the GI tract deficient in free thymine or thymidine. A Phase I clinical trial using this strain showed a significant decrease in disease activity in Crohn's patients with only minor adverse effects. Additionally, the bacteria did not survive passage through the GI tract, indicating that the containment strategy was effective [96]. Significant barriers remain, however, to the widespread application of this approach. Various issues include bacterial toxicity of some antigens, failure of bacteria to properly glycosylate glycoproteins, intrinsic variability of immunoadjuvant properties in different probiotic strains and whether or not persistence or transient passage of the microbe designed to deliver the antigen is optimal.

Expert commentary

Long-lasting protection against diverse pathogenic microbes that have penetrated into the mucosa requires robust B- and T-cell immune responses. The latter are generated by proper activation of professional APCs and cytokines released into the microenvironment. To achieve effective immunity within the mucosal site, the challenge for any researcher has now become optimization of vaccine strategies that lead to efficient targeting and delivery of immunogens to mucosal DCs. This must be done via proper activation with an adjuvant that is tolerable for human use. Moreover, employing such a strategy with the necessary tools should elucidate protective immune correlates against pathogenic microbes that include triggering the right mucosal DC subsets and balancing their activation by adjuvants, enhancing the longevity of mucosal Th1, Th2 and Treg cells and, finally, activating B cells that secrete effective sIgA against microbes.

Five-year view

Various vaccine strategies and immunomodulatory agents, such as adjuvants, are currently used in several preclinical settings. However, very few vaccine strategies have been approved by FDA. This clearly indicates that more hard work on the optimization of vaccine formulations is still required for passing the many hurdles evident, before human use. Additional tools, including receptor-targeting methods and the adjuvant properties of agonistic plants, microbial products and/or Grampositive bacteria, such as lactobacilli, should be explored to further refine the desired immunity necessary for its induction.

Financial & competing interests disclosure

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Key issues

- Mucosal surfaces represent the major portal of entry for pathogens, a home for commensal bacteria, and contain the highest concentration of lymphocytes found throughout the body.
- The mucosal immune regulation is directed through an integrated network of specialized immune cells, including dendritic cell (DC) subsets and macrophages via various costimulatory and coinhibitory signals that impact the function of B- (production of secretory IgA) and T-cell subsets (Th1, Th2, Th17 or Treg cells).
- DCs are an important sentinel arm of the immune system that induces either costimulatory or regulatory immune responses. Such action determines the type of humoral and T-cell-mediated immune responses in humans.
- *Lactobacillus* spp. are common members of the human gastrointestinal microbiota. The highest levels of lactobacilli are typically found in the small intestine.
- Various *Lactobacillus* spp. have been consumed by humans in large numbers as probiotic cultures, ingested either as dietary supplements or in more traditional fermented milk products, such as yogurt.
- Some of the benefits attributed to consuming probiotic cultures include protection against enteric pathogens and viruses, tolerance to lactose, anticarcinogenic properties, antiallergenic protection and modulation of the immune system.
- Probiotic bacteria, especially specific *Lactobacillus* strains (e.g., *Lactobacillus acidophilus*), are not only efficient vectors for vaccine delivery but also serve as potent adjuvants. These microorganisms can induce a balanced inflammatory microenvironment via DC and macrophage activation.
- Mucosal immunization induces not only mucosal but also systemic immunity, which effectively prevents infection.

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